Optimization of *In vitro* Micropropagation, Characterization of Bioactive Compounds and Biological Activities of *Cynanchum tunicatum* (Retz.) Alston – A Rare Medicinal Plant

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Cynanchum tunicatum (Retz.) Alston belongs to the family Apocynaceae, encompassing roughly 378 genera and approximately 5,350 species, predominantly distributed across pantropical regions, this genera extending into temperate zones and it is commonly known as Dog Strangling Vine or Milkweed (Endress et al., 2018) . Various Cynanchum species have potential medicinal properties addresses fever, cough, pneumonia, asthma and various ailments. Literature surveys indicate approximately 450 compounds from diverse Cynanchum species, which include steroids, alkaloids, terpenes, flavonoids, polysaccharides, and steroidal glycosides (Han et al., 2018). Based on previous research, other Cynanchum species have potential secondary metabolites. Henceforth, to explore the medicinal properties of *C. tunicatum*, in this present study phytochemical profiling were analyzed yet there is no previous phyto-constituents were reported.

There is no scientific evidence for this *C. tunicatum*, this present study is the novel scientific report for the phytochemical and biological activities such as antimicrobial, antioxidant, anticancer and anti-inflammatory. Since ancient period, indigenous people using *C. tunicatum* as folk medicine, so this plant species become RET listed. For conserving this species, *in vitro* micropropagation was standardized.

Plant tissue culture techniques, a subset of biotechnological tools, have successfully contributed to two key areas: the commercial production of microbe-free plants and the preservation of germplasm for rare and endangered plant species (Sharma et al., 2021; Syeed et al., 2021). Conservation is required for *C. tunicatum* because it extinct by the indigenous people for their folk medicine to many ailments and since now there is no scientific evidence. In natural condition the seed germination percentage was very low (10%), to overcome the germination barrier using the *in vitro* propagation method. To optimize the abiotic factors for seed germination through the *in vitro* propagation, with the help of two factorial integration models (2 FI) of response surface methodology (RSM).

In this present study, provides the macroscopic characteristics of *C. tunicatum*. It exposes its perennial nature, twinning growth pattern, and distinctive morphological features such as heart-shaped leaves, white latex secretion, and pedunculate flowers forming umbels, the fruit is follicle that releases seeds through a suture, splitting open along a seam to disperse its seeds. The macroscopic examination attributes valuable insights into its taxonomy and ecological role. It enhanced our understanding of this species, contributing to its conservation.

Microscopic observation plays a pivotal role in plant anatomy, serving as a bridge between traditional plant sciences and modern techniques in micrometry studies. It provides essential insights

into plant meristems, cell differentiation, and tissue dynamics, foundation for developmental biology (Sonibare & Adebodun, 2018).

The significance of microscopic observation in unraveling the intricacies of plant anatomy of *C. tunicatum*. Its historical importance and contemporary relevance highlight its enduring value as a fundamental aspect of plant science. By shedding light on plant meristems, cell differentiation, tissue dynamics, and microscopic observation enriches of this plant evolution and development, paving the `way for further exploration and comparative study of *in vitro* callus, organogenesis differentiation.

In this present study, *in vitro* callus development, direct organogenesis, indirect organogenesis and micropropagation were standardized by tissue culture techniques were discussed.

An effective surface sterilization method was used to remove dust particles and microbes from explants. The *in vitro* seed germination of *C. tunicatum* were sterilized using various concentrations of sodium hypochlorite ($NaHCl_3$) and mercuric chloride ($HgCl_2$). Surface sterilization is one of the crucial factors, to prevent contamination and ensure successful *in vitro* seed germination. Established standardized protocol for *in vitro* propagation techniques, facilitating further experimentation and application of conservation method.

Using Response Surface Methodology (RSM), this study investigated the germination rate of *C. tunicatum* seed. Seeds were inoculated on MS medium without plant growth hormone. Achieved a remarkable 96% germination rate, influence of the three abiotic factors – pH (5.8), photoperiod (16/8 hours), and 3% sucrose concentration played significant role on germination process on the controlled condition. The results obtained from the optimal ranges for these parameters on MS basal medium without plant growth regulators. This knowledge contributes to the advancement of plant tissue culture techniques, aiding in the conservation and sustainable utilization of this species. Further investigation into these parameters could lead to continued improvements in seed culture methodologies and the production of healthy plantlets for conservation.

Embryo culture techniques were applied using the standardized protocol of abiotic factors for *C. tunicatum*. Mature embryos were isolated from seedlings and cultured on MS basal medium without plant growth hormone. *In vitro* embryos showed 100% germination at 15^{th} days of post-inoculation and complete shoot formation achieved by 80^{th} day. The efficacy of embryo culture highlights the efficient protocol to foster the germination percentage, shoot and root development. The germination rate (100%) emphasizes the viability and efficiency of this technique in micro-propagation for *C. tunicatum*. It contributes the advancement of plant tissue culture methodologies and hold promise for

the conservation and propagation of this species. Further research could explore the scalability and application of this technique for large-scale propagation.

Optimization of callus induction in *C. tunicatum* leaf explants through a series of experiments varying hormonal concentrations and combinations on MS medium. Fifteen combinations were examined, resulting in the dedifferentiation of parenchymatous cells and the formation of friable calli. The maximum percentage of callus induction showed 89.43% in the concentration of 2 mg L^{-1} 2,4- D + 2 mg L^{-1} IAA amended with MS basal medium. The formation of calli emphasized the importance of hormonal balance, particularly the synergistic effect observed with IAA and 2,4-D.

Callus induction of nodal explants of *C. tunicatum* on MS medium with various plant growth regulators, observing both callogenesis and organogenesis. Explants were inoculated with cytokinin such as BAP which led to the formation of multiple shoots, while auxins such as IAA, IBA, NAA, and 2,4-D prompted the development of friable calli. The maximum percentage of callus induction in nodal explant showed 85.64 % in the concentration of 2 mg L^{-1} of 2,4 D + 2 mg L^{-1} of IAA. The above-mentioned combinations and concentrations of the hormones have the dual response of callus induction as well as organogenesis from the nodal explants. The significant biomass was obtained from the callus attained in the specific hormonal combinations emphasizes the importance of optimizing culture conditions.

The *in vitro* internodal explants of *C. tunicatum* demonstrated callus proliferation on 15th days of inoculation. MS basal medium fortified with 2.0 mg L^{-1} of 2,4 D + 2.0 mg L^{-1} of IAA enhanced callus induction, resulting in the highest percentage of 90.39 %. Various concentrations of BAP supplemented with MS media alone responsible for different color and texture of calli for instance, light brown compact calli were observed.

The maximum callus induction observed in root explants on MS medium supplemented with 2.0 mg L^{-1} of 2,4-D + 2.0 mg L^{-1} of IAA. On the 15th day of inoculation, explants were differentiated to form undifferentiated parenchymatous cells. This approach facilitated successful and early cell proliferation, leading to robust callus initiation. The highest percentage of callus (94.57 %) was achieved with the combination of 2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} IAA.

The rapid callus proliferation observed in all explants exemplified the potential of specific hormonal combinations enhanced culture efficiency. Root explants exhibited superior callus formation potential under the optimized *in vitro* protocol for *C. tunicatum*. The rapid initiation of callus induction obtained the efficiency of the selected hormonal combinations.

Somatic embryogenesis in *C. tunicatum* was achieved from root-derived callus on MS

medium supplemented with 1.0 mg L^{-1} 6-BA and 1.5 mg L^{-1} 2,4-D. Morphological changes in cells were observed from irregular to ovate shape on 25th day transformed to globular shape then developed into heart shaped on 40th day. After the 45th day the narrow portion elongated to form a torpedo shaped and cotyledonary somatic embryos were observed on 50th day. Somatic embryos were transferred to MS medium containing 1.5 mg L^{-1} 2,4-D and 1 mg L^{-1} TDZ. Somatic embryogenesis observed in *C. tunicatum* offers avenues for mass propagation.

In vitro indirect shoot proliferation of *C. tunicatum* was achieved on MS medium supplemented with 6-BA and TDZ, with the highest shoot production (93.3%) observed at 0.5 mg L^{-1} 6-BA and 2 mg L^{-1} TDZ on 70th day.

Multiple shoots were regenerated from *C. tunicatum* on MS medium with 6-BA and TDZ. The regenerated shoots were transferred to rooting media containing various growth hormones. Root formation was achieved on MS medium supplemented with 2.0 mg L^{-1} IBA (97.6%) on 85th day. *In vitro-grown* plants were acclimatized in a hyco-pot with vermiculate and sterile soil, then transferred to earthen pots and planted in the field, 100% survival in 6th week was observed. Efficient root formation and acclimatization protocols are crucial for the transfer of *in vitro* regenerated plants to field conditions. The high survival rate after transfer highlights the robustness of the developed protocol, offering a reliable method for the mass propagation and field establishment of *C. tunicatum* plants.

Nodal explants of *C. tunicatum* inoculated on MS basal medium supplemented with 1 mg L^{-1} of BAP and 1.5 mg L^{-1} of 2,4-D, for direct *in vitro* organogenesis. The proliferation of adventitious shoots was observed in three weeks of culture. Further subcultured on MS medium with 2 mg L^{-1} IBA for inducing root development and it showed 100% germination rate in *C. tunicatum* obtained on four weeks.

Histological analysis of *C. tunicatum* was carried out in the *in vitro* derived somatic embryos to distinguish the stages of embryogenesis and organogenesis. Primordial cell clumps and intensive cell divisions were observed in embryonic calli. Non-embryogenic calli displayed irregular parenchymatous cells with visible intercellular spaces. Compact meristematic cells, somatic pro-embryos with suspensor-like structures, developed globular-shaped somatic embryos were observed. Shoot and root formation, as well as anatomical features like endodermis and stomatal pores, were observed. The observed stages of embryogenesis and organogenesis in *C. tunicatum* provide valuable insights into its regeneration processes. Understanding these mechanisms can inform the refinement of tissue culture protocols for further propagation and biotechnological applications in *C. tunicatum* and related species.

In vitro shoots and roots of *C. tunicatum* were detached from the culture medium, washed with sterile distilled water to remove agar, and transferred to plastic pots filled with soil and organic manure (3:1 ratio). They were kept in a greenhouse for 15–25 days before being transferred to mud pots, where they exhibited a 100% survival rate. Successful acclimatization of *in vitro* plantlets from culture medium to greenhouse conditions, followed by transfer to mud pots, resulted in a high survival rate. This protocol demonstrates a reliable method for transitioning plants from tissue culture to field conditions, facilitating further growth and development.

An ethnobotanical survey in the Sirumalai forest, Dindigul district, revealed the local use of *C. tunicatum* for treating microbial and viral infections. The plant powder is characterized by its coarse texture, light green color, light odor, and sweet taste, with a rough feel when touched. The findings from the ethnobotanical survey shed light on the traditional medicinal uses of *C. tunicatum* in the Sirumalai forest region. The organoleptic study provides valuable insights into the physical characteristics of the plant powder, aiding in its identification and potential medicinal application.

Preliminary phytochemical analysis of wild plant and callus extracts of *C. tunicatum* was carried out by sequential methods using organic solvents such as hexane, chloroform, ethyl acetate, and methanol. Ethyl acetate and methanolic extracts from both sources revealed abundant secondary metabolites, including alkaloids, carbohydrates, glycosides, phenols, terpenoids, quinines, and phyto steroids. The presence of diverse secondary metabolites in *C. tunicatum* extracts, particularly in ethyl acetate and methanol extracts from both field-grown and callus sources. These findings provide a foundation for further investigation into the pharmacological potential and biochemical composition of *C. tunicatum*.

Total alkaloid content was quantified in various extracts such as PHE (Plant Hexane Extract), PCE (Plant Chloroform Extract), PEAE (Plant Ethyl Acetate Extract), PME (Plant Methanolic Extract), CHE (Callus Hexane Extract), CCE (Callus Chloroform Extract), CEAE (Callus Ethyl Acetate) and CME (Callus Methanolic Extract) of *C. tunicatum*. The highest alkaloid content was observed in the PME extract (1.37±0.03 µg/mL) and CME extract (1.074 µg/mL) of *C. tunicatum*. Total flavonoid content was quantified in various solvent extracts of *C. tunicatum* using the aluminum chloride method. The yellow color indicated the presence of flavonoids, with the highest content observed in the PME extract (3.58±0.02 µg/mL), followed by the CME extract (3.36±0.05 µg/mL).

The phenolic content of *C. tunicatum* was determined in PME and CME extracts, with values of 2.32±0.037 µg/mL and 3.80±0.08 µg/mL, respectively. The higher phenolic content observed in the CME extract compared to the PME extract. Phenolic compounds are known for their antioxidant

properties, indicating the potential health benefits. Among the extracts tested, PME exhibited the maximum terpenoid content at 1.60±0.02 µg/mL, followed by PEAE at 1.57±0.14 µg/mL.

The significant amount of secondary metabolites observed in PME and CME extracts suggests that these extracts are rich sources of secondary metabolites in *C. tunicatum*. These are known for their diverse pharmacological activities, indicating the potential therapeutic significance of *C. tunicatum* extracts in traditional medicine and pharmaceutical applications.

The FTIR analysis of *C. tunicatum* revealed the presence of various functional groups including alkaloids, flavonoids, phenols, terpenoids, carboxylic acid, alkane, and aromatic alkenes in both wild plants and callus extract. The comprehensive FTIR analysis underscores the diverse chemical composition of *C. tunicatum*, spanning alkaloids to aromatic alkenes. These results provide valuable insights into its potential medicinal and therapeutic applications.

GC-MS analyses of PME and CME extracts from *C. tunicatum* obtained 45 compounds. Major compounds identified such as 1,3-Dioxolane, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 9,12-Octadecadienoic acid, 9-Hexadecenoic acid, Octadecanoic acid, Tetradecanoic acid, and l-(+)-Ascorbic acid 2,6-dihexadecanoate. Further research into the biological activities of these compounds could elucidate their therapeutic potential and contribute to the development of novel drugs or nutraceuticals industries.

Alkaloids, and terpenoids in *C. tunicatum* were identified and quantified using standards such as colchicine, and rutin. The colchicine was observed as 0.12 µg and 0.033 µg in 1 µL of PME and CME respectively. The rutin was observed as 0.211 µg and 0.12 µg in PME and CME respectively. This analytical approach facilitated the accurate determination of these bioactive compounds in the plant. By employing standards for comparison, the identification and quantification of alkaloids, and flavonoid in *C. tunicatum* were achieved with precision.

The antibacterial activity of CME from *C. tunicatum* was assessed using the well-diffusion method against two gram-positive bacteria (*Salmonella enterica* and *Enterococcus faecalis*) and one gram-negative bacteria (*Escherichia coli*). Amphicilin and DMSO served as positive and negative controls, respectively. CME exhibited strong antibacterial activity, with a maximum inhibition zone of 32.5±1.8 mm observed against *Salmonella enterica* at a concentration of 100 µL. Followed by another pathogenic bacteria *Enterococcus faecalis* showed the maximum zone of inhibition (15.2±1.4 mm) at the concentrations of 100 µL. *Escherichia coli* inhibits the maximum zone of 13.8±1.2 mm at concentrations of 100 µL.

The anti-fungal activity of Plant Methanolic Extract (PME) from *C. tunicatum* was investigated against *Aspergillus niger* and *Candida albicans*. the highest inhibition zone of 15.3±0.9 mm was observed at the concentration of 100 µL, 12.8±1 mm in 75 µL, 11.8±1 mm in 50 µL and 12.4±0.5 mm in 25 µL. After that, the maximum inhibition zone was observed (10.9±1.1 mm) at the concentration of 100 µL and 10.1±0.8 mm (75 µL) against the *Candida albicans.*

The values for MIC and MBC of four different extracts against five microbes including three bacteria and two fungi. The MIC value of CME against *Salmonella enterica* was 25 µg/mL and PEAE against *Aspergillus niger* was 25 µg/mL. On the other hand, the PEAE showed MBC at the concentration of 100 µg/mL against *Enterococcus faecalis*. The PEAE of MFC values showed 75 µg/mL in all extracts and the CEAE showed no activity against *Candida albicans*.

The antioxidant activity was analyzed for all four crude extracts of *C. tunicatum* using DPPH assay. The maximum inhibition percentage was obtained in PME (78.73%) whereas standard ascorbic acid showed 85.9%. Followed by CME (84.08%), PEAE (69.11%), and CEAE (70.82%). The Half-minimum inhibitory concentration was examined as PME (IC_{50} = 53.1 µg/mL), CME (IC_{50} = 52.2 µg/mL), PEAE (IC_{50} = 76.15 µg/mL), and CEAE (IC_{50} = 63.4 µg/mL). The maximum FRAP value was observed at a concentration of 100 µg/mL in CME (1.6 µg/mL), CEAE (1.09 µg/mL), PME (1.27 µg/mL) and PEAE (0.98 µg/mL). The ferric-reducing antioxidant power observed significantly in CME and CEAE of *C. tunicatum*.

The ABTS assay was performed in all the four extracts PEAE, PME, CEAE and CME of *C. tunicatum*. The percentage scavenging activity of all four extracts showed CME (76.34%), followed by CEAE (65.5%), PME (70.77 %) and PEAE (56.42%) was observed in the concentration of 100 µg/mL. The standard ascorbic acid showed 82.24%. The IC_{50} values of PEAE, PME, CEAE, and CME showed 99.58, 72.99, 75.73, 60.24 and 51.39 µg/mL respectively.

The total antioxidant activity was observed at a concentration of 100 µg/mL in CME (83.68%) followed by PME (81.83%), CEAE (62.19%) and PEAE (58.63%). The IC_{50} values of PEAE, PME, CEAE, CME and AA showed 82.11, 47.11, 86, 53.38 and 32.91 µg/mL respectively. These results signify the significance of natural antioxidant research and its potential applications in combating oxidative stress-related ailments. Further exploration of the antioxidant mechanisms and identification of key bioactive compounds could lead to the development of novel antioxidant therapies.

The anti-inflammatory activity of four different extracts was evaluated by protein denaturation assay. The maximum inhibition percentage of CME (310.74%), PME (237.56%), CEAE (272.46%) and PEAE (130.81%). Result showed the IC_{50} value of PEAE (44.94 µg/mL), PME (22.3 µg/mL),

CEAE (25.7 µg/mL), CME (23.08 µg/mL) and Diclofenac sodium (29.91 µg/mL). *C. tunicatum* extracts, particularly CME and PME, demonstrate potent inhibition of protein denaturation, highlighting their potential as natural protectants against oxidative damage.

The cell viability and cytotoxicity of CME were evaluated against the human colorectal carcinoma cell line (HCT-116) at concentrations ranging from 1.56 to 50 µg/mL. The highest percentage of cell viability for CME was observed at 1.56 µg/mL (91.87%), followed by a gradual decrease at higher concentrations. The IC_{50} value for CME was determined to be 16.71 µg/mL. The study demonstrates the potential cytotoxic effects of CME against HCT-116 cells, indicating its possible utility as an anti-cancer agent and paving the way for potential anticancer treatment, particularly colorectal carcinoma.

The Brine Shrimp Lethality Assay was carried out to evaluate the toxicity of *C. tunicatum* extracts. Among all extracts, CME exhibited the lowest toxicity, with a mortality rate of 53% observed after 24 hours at the concentration of 1500 µg/mL. The lethal rate showed 17%, 20%, 27%, 40%, and 53% increased proportionately with increasing drug concentration of 100, 250, 500, 1000, and 1500 µg/mL were observed in CME, respectively. Additionally, CEAE showed the second lowest mortality rate, with percentages of 10%, 17%, 40%, 16%, and 60% observed in 100, 250, 500, 1000, and 1500 µg/mL respectively. CME extracts of *C. tunicatum* exhibited there is no evidence of toxicity even in the higher concentrations.

Docking studies are crucial in drug discovery for predicting ligand-receptor interactions and ranking compounds based on binding energies or fitness scores. In this present study, multiple compounds were docked with the primary drug pathway for the HCT-116 cell line and determined their fitness scores. The compounds were ranked by their fitness scores. The highest-scoring compound was then compared with ligand and protein (6 GUE) to analyze its binding pattern and molecular interactions. Compounds showed highest binding affinity against 6 GUE. The designed compounds formed the amino acids fractions such as phenylalanine (PHE82, PHE80), glutamic acid (GLU81), alanine (ALA31, ALA144) (VAL64, VAL18), lysine (LYS33), aspartic acid (ASP145), leucine (LEU134) and isoleucine (ILE10) of the CDK_2 enzyme.

Cynanchum tunicatum promises combating bacterial and fungal infections. Antibacterial and anti-fungal mechanisms provide scientific knowledge for the development of novel antimicrobial therapies. Antioxidant properties and its potential applications in functional foods or pharmaceuticals aimed at reducing oxidative stress-related diseases. Antioxidant activity exhibits the therapeutic relevance in preventing protein damage caused by harmful pathogens or free radicals. These results

suggested that potential safety profile of CME for pharmaceutical applications. Further investigation is required for the pharmacological activities and specific bioactive compounds were acknowledge for novel drug discovery.

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